ELSEVIER

European Journal of Pharmacology 410 (2000) 15–23

ejp

www.elsevier.nl/locate/ejphar

Effects of arginine vasopressin in the heart are mediated by specific intravascular endothelial receptors

Tania Zenteno-Savin [a,1], Isabel Sada-Ovalle [a], Guillermo Ceballos [b], Rafael Rubio [a,*]

[a] *Departamento de Fisiologia y Farmacologia, Facultad de Medicina, UASLP, Av. V. Carranza # 2405, Col. Los Filtros, San Luis Potosi, SLP, CP 78210, Mexico*

[b] *Escuela Superior de Medicina, IPN, Mexico, DF, Mexico*

Received 10 October 2000; accepted 2 November 2000

Abstract

Arginine vasopressin induces vascular, inotropic and arrhythmogenic effects in the heart. Existing evidence, obtained indirectly, suggests that these effects occur through paracrine endothelial mechanisms. To demonstrate this, vasopressin was confined to the intravascular space by covalent coupling to high molecular weight (2×10^6 Da, vasopresin–dextran) dextran. Isolated guinea pig hearts were infused with equivalent concentrations of vasopressin and vasopressin–dextran. The negative inotropic and coronary vasopressor effects of vasopressin–dextran were similar to those evoked by vasopressin; in both cases effects were reversible. Free dextran had no effect on vascular resistance nor in ventricular developed pressure. The inotropic and vascular effects of both vasopressin and vasopressin–dextran were blocked by the vasopressin receptor antagonist [Adamantaneacetyl[1], *o*-Et-D-Tyr[2], Val[4], Aminobutyryl[6], Arg[8,9]]vasopressin (Adam–vasopressin), indicating that the effects of the two agonists were vasopressin receptor-mediated. To elucidate possible endothelial intermediaries of these effects, isolated guinea pig hearts were infused simultaneously with vasopressin or vasopressin–dextran and several inhibitors either of synthesis or blockers of receptors of possible endothelial mediators. Only reactive blue 2, a P_{2y} purinoceptor antagonist, and suramin, a P_{2y} and a P_{2x} purinoceptor antagonist, caused a total reversal of vascular and inotropic effects of vasopressin and vasopressin–dextran. Pyridoxalphosphate-6-Azophenyl-2′-4′disulphonic acid, a P_{2x} purinoceptor antagonist, was without effect. Our results provide direct evidence that the short-term cardiac effects of vasopressin are due to selective activation of intravascular purinoceptors and suggest that an intermediary of these effects is ATP.

Keywords: Hormonal control; Endothelial receptor, luminal; ATP; Purinoceptor; Endothelial mediator; Cardiac function control

1. Introduction

Blood pressure and osmoregulation are considered to be the main functions of the vasopressin system. The action of vasopressin is mediated through activation of specific membrane-bound, V_1—vascular and V_2—renal subtypes (Michell et al., 1979). Vasopressin receptors are widely distributed and its actions include vasoconstriction and antidiuresis (Swords et al., 1991). However, the peptide may also participate in other functions such as promoting cell growth and proliferation (Moalic et al., 1989; Murase et al., 1992), cardiac function regulation and perfusion and neurohormone secretion (Cowley and Liard, 1987; Graf et al., 1997; Hupf et al., 1999; Marttila et al., 1996). In the heart, vasopressin causes dose-dependent coronary constriction and myocardial depression at physiologic concentrations (Boyle and Segel, 1986, 1990) and has a direct negative inotropic effect (Chiba, 1977; Wilson et al., 1980). Recently, a paracrine cardiac vasopressin system, including vasopressin mRNA, was identified in rat heart (Hupf et al., 1999). Furthermore, it has been shown that the endothelium plays an important role in modulating the vascular responses to vasopressin (Katusic et al., 1984). However, it is unknown if the cardiac effects of vasopressin are also endothelium-mediated. To demonstrate that in the intact heart the effects of vasopressin on various cardiac functions are exerted through an endothelium-mediated paracrine mechanism, vasopressin would have to be confined to the intravascular space. To prevent the diffusion of

* Corresponding author. Tel.: +52-48-26-23-45 x 254; fax: +52-48-26-23-55.

E-mail address: rrubio@deimos.tc.uaslp.mx (R. Rubio).

[1] Actual address: CIBNOR, La Paz, Baja California Sur, Mexico.

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00853-0

intracoronarily administered vasopressin, we have covalently coupled this polypeptide to high-molecular weight (2×10^6 Da) dextran, and the inotropic and vascular effects of vasopressin bound to dextran (vasopressin–dextran) and free vasopressin were compared in isolated guinea pig hearts. The results provide direct evidence that these short-term effects of vasopressin are due to selective activation of intravascular receptors and suggest that the intermediary of these effects is ATP.

2. Materials and methods

2.1. Isolated saline-perfused hearts

Male English short-hair guinea pigs (350–400 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and heparin sodium (500 U). The animals were artificially ventilated, the chests were opened, and a loose ligature was passed through the ascending aorta. The hearts were rapidly removed, immersed in ice-cold physiological saline, retrogradely perfused via a non-recirculating perfusion system at constant flow, and trimmed of non-cardiac tissue. Coronary flow was adjusted with a variable-speed peristaltic pump (Harvard Apparatus, model 1215). An initial perfusion rate of 25 ml/min for 5 min was followed by a 25-min equilibration period at a perfusion rate of 10 ml/min. After this period, experiments were begun and all hearts were perfused at a coronary flow of 10 + ml/min and this flow was maintained throughout the experiment. The perfusion medium was Krebs–Henseleit solution with the following composition (mM): 117.8 NaCl, 6 KCl, 1.75 $CaCI_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 24.2 $NaHCO_3$, 5 glucose and 5 sodium pyruvate. The solution was equilibrated with 95% O_2–5% CO_2, pH adjusted to 7.4, and kept at 37°C. Since all experiments were performed at a constant coronary flow, the coronary vascular resistance was estimated from measurements of the coronary perfusion pressure which was recorded continuously via a side arm of the perfusing cannula.

A pair of stimulating electrodes, made of small stainless steel wire vascular clamps (Fine Surgical Instruments) soldered to a thin flexible wire, were placed 2 mm apart in the apex of the right atrium. Pacing was achieved by applying electrical square pulses of 2-ms duration and twice the electrical threshold at a rate of 5.1 ± 1.1 Hz.

To measure ventricular pressure development via the left atrium, a fluid-filled latex balloon was introduced into the left ventricle. Diastolic pressure was adjusted to about 10 mmHg and the developed pressure continuously determined. The pressure developed under control conditions was defined as 100% and all other amplitudes measured under the infusion of either vasopressin or vasopressin–dextran conditions were expressed as percent of control.

2.2. Coupling vasopressin to high-molecular weight dextran

The procedure used to couple vasopressin to high molecular weight dextran was that published by Haga and Haga (1983) and modified by the authors (Ceballos and Rubio, 1998; Rubio et al., 1999). Briefly, 1 g of dextran (2×10^6 Da) was dissolved in 50 ml of 0.1-M sodium phosphate buffer (pH 7.4) and 1.18 g $NaIO_4$ (5.55×10^{-3} mol). This solution was mixed and stirred continuously for 3 h on ice. The reaction caused oxidation of hydroxyl groups in the two adjacent carbon atoms of every glucose moiety of the dextran, forming a pair of reactive aldehyde endings. To this solution 1.44 g of 6-aminocaproic acid was added, causing the formation of a Schiff base (between the amino group of aminocaproic acid and the aldehyde endings of the dextran). The reaction was allowed to proceed 4 h with continuous stirring at room temperature. Thereafter, to reduce and stabilize the Schiff base, 0.9 g of $NaCNBH_3$ was added and the reaction proceeded overnight at room temperature in the dark. The final solution was dialyzed against several changes of distilled water, precipitated with ice-cold acetone, and dried. The product formed, dextran–aminocaproic acid, had an estimated 4.9×10^6-Da molecular weight. The dextran–aminocaproic acid powder was dissolved in 50 ml of 0.1-M sodium phosphate buffer (pH 7.4) and divided into three equal aliquots. To one of the aliquots no other addition was made (control 1). To the second aliquot, 1.5 g of [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] was added, in order to activate the free carboxylate group of aminocaproic acid (Detar and Silverstein, 1966); the solution was incubated with constant stirring for 30 min. Afterwards, 10-mg vasopressin were added and incubation continued for 4 h at room temperature before adding another 1.5 g of [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide]; the reaction was allowed to proceed overnight to form the complex vasopressin–dextran with an estimated molecular weight of 2.5×10^7 Da. To the third aliquot vasopressin was added, with no [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] (control 2); the objective of this solution was to check for non-covalent binding of vasopressin to the dextran. The three different aliquots were precipitated with ice-cold acetone, resuspended in distilled water, dialyzed against several changes of water, and lyophilized. Stock solutions were prepared with distilled water and kept frozen at −20°C. Immediately before the experiment, working solutions were prepared with Krebs–Henseleit buffer equilibrated with 95% O_2–5% CO_2, pH adjusted to 7.4, and temperature at 37°C.

2.3. Effects of control dextran–aminocaproic complexes 1 and 2

In isolated perfused guinea pig hearts, a sustained intracoronary infusion of one-dextran–aminocaproic complexes

(controls 1 and 2) was assayed at a final concentration of 1×10^{-7} M. The ventricular contraction and perfusion pressure were continuously monitored and plotted against time of infusion. These experiments were designed to rule out any effect of the dextran–aminocaproic alone (control 1) or non-covalent binding of vasopressin to dextran–aminocaproic (control 2). The 10^{-7}-M concentrations of dextran–aminocaproic controls were at least three orders of magnitude higher than the concentration of vasopressin–dextran required to cause maximal inotropic and vascular effects. There were six hearts for each of these two control groups.

2.4. Possible hydrolysis of vasopressin–dextran complex during its passage through the coronary circulation

To rule out the possibility that the vasopressin–dextran complex became hydrolyzed to free vasopressin during its passage through the heart, 50 ml of venous effluent were collected during a 5-min infusion of vasopressin–dextran (10^{-11} M) and was filtered in a Centrifugal Ultrafree (Millipore) unit (MW cut-off = 10,000 Da) by centrifuging at $1500 \times g$ for 15 min. The filtrate (control 3), that is, the low molecular weight fraction (MW < 10,000 Da) which would contain free vasopressin, was equilibrated with 95% O_2–5% CO_2 and brought to 37°C. The solution was perfused into the same heart preparation and the perfusion pressure and ventricular contraction were continuously recorded ($n = 6$).

2.5. Bioassay of the vasopressin–dextran complex and determination of equipotency with vasopressin

Bioassay system utilized was the isolated thoracic aorta of guinea pigs. The aorta was excised, placed on a petri dish with Krebs–Henseleit buffer and cleaned of connective and adipose tissue. Rings, 1–2-mm-wide, were cut preserving the intactness of the endothelium. Using two small stainless steel wire hooks, each ring was mounted vertically on an isolated tissue chamber filled with 10 ml of high-K^+ (20 mM) Krebs–Henseleit buffer, equilibrated with 95% O_2–5% CO_2, pH adjusted to 7.4 and temperature at 37°C. One hook was connected to a force displacement transducer (Grass) and its output was recorded on a pen recorder (Gould). The other hook was attached to a micrometer used for changing muscle length. Aortic rings were stretched 50% of their slack length and allowed to equilibrate for at least 1 h before experiments were begun. The active tension developed during K^+ depolarization activation (124 mM K^+) at several different lengths was measured to determine the length (L_0) at which maximal force was developed (Herlihy et al., 1976). The contraction stimulating effects of vasopressin and vasopressin–dextran were determined at L_0 (3.5 ± 0.6 mm; $n = 20$).

2.5.1. Comparison of potencies

In order to compare the potency of vasopressin–dextran with that of free vasopressin, their dose–response curves were determined in the same aortic ring. The molecular weights estimated for vasopressin and vasopressin–dextran were 1.08×10^3 g/mole and 2.5×10^7 g/mole, respectively. Dose–response curves were obtained by adding increasing amounts of vasopressin (range: 10^{-10}–10^{-7} M) or vasopressin–dextran (range: 10^{-14}–10^{-11} M) to the bath and plotting developed tension against concentration (data not shown). From these dose–response curves the molar concentration of vasopressin–dextran needed to cause the same effects as a given concentration of vasopressin were assessed. It was determined that vasopressin–dextran required a concentration equal to [vasopressin]/10^4 to produce the same effect as [vasopressin]. We defined $1/10^4$ as the molar equivalent activity of vasopressin–dextran as compared with that of vasopressin. The determined concentration ratio [vasopressin]/[vasopressin–dextran] = 10^4 is a reasonable figure, since the estimated maximal number of bound vasopressin moieties per dextran molecule is 2.2×10^4, i.e. this indicates that if all potential sites for binding for vasopressin were saturated, our compound behaves as if only 0.454($0.454 \times 2.2 \times 10^4 = 1 \times 10^4$) of the total bound vasopressin is active.

2.6. Effects of vasopressin and vasopressin–dextran on guinea pig isolated hearts

After the molar equivalency of vasopressin and vasopressin–dextran was determined, effects of vasopressin and vasopressin–dextran were assessed in isolated guinea pig hearts perfused at constant flow. For the dose–response effects, five concentrations of vasopressin were chosen: 10^{-10}, 10^{-9}, 5×10^{-9}, 10^{-8} and 5×10^{-8} M. Three-minute infusion of a dose of AVP lasted 3 min and was followed by a washout period of 15 min with Krebs–Henseleit buffer alone. This washout period was followed by a 3-min infusion of an equivalent concentration of vasopressin–dextran (range: 10^{-14}–10^{-11} M), followed by a 15-min washout period. This cycle was repeated by another dose. Thus, there was an 18-min interval between the beginning of a dose to the beginning of the next one. This program of infusion yielded reproducible dose–response curves. Perfusion pressure and ventricular contraction were continuously recorded.

2.7. Vasopressin receptor blockade

To determine that the cardiac inotropic and vasoconstriction effects of vasopressin–dextran were mediated via the vasopressin receptor, the polypeptide vasopressin receptor antagonist, [Adamantaneacetyl^1, *o*-Et-D-Tyr^2, Val^4, Aminobutyryl^6, Arg^{8,9}]vasopressin (Adam–vasopressin, Manning et al., 1987) was continuously intracoronarily

infused at a maximal concentration (10^{-6} M) for 10 min prior the administration of the highest vasopressin or vasopressin–dextran concentrations tested. Thereafter and during the sustained infusion of Adam–vasopressin, vasopressin (5×10^{-8} M) or vasopressin–dextran (5×10^{-12} M) were infused for a 3-min period ($n = 3$) as described above.

2.8. Inhibition or blockade of possible endothelial mediator mechanisms

To determine the possible mediators of the inotropic and vascular effects of vasopressin and intravascular vasopressin–dextran, several inhibitors were infused in parallel to infusions only with the largest tested doses of vasopressin (5×10^{-8} M) or vasopressin–dextran (5×10^{-12} M). The infusion period lasted 3 min. The receptor inhibitors tested were: three purinoceptor antagonists—reactive blue 2 (2×10^{-6} M), a P_{2y} receptor antagonist (Fieber and Adams, 1991); pyridoxal-phosphat-6-azophenyl-2′,4′-disulphonic acid (5×10^{-5} M), a P_{2x} receptor antagonist (Zinganshin et al., 1993); and suramin (7.5×10^{-5} M), a P_{2y} and P_{2x} antagonist (Ohno et al., 1993; Anning et al., 1999), and aminophylline (2×10^{-5} M), a blocker of adenosine receptors (Harden, 1991). The enzymatic inhibitors tested were: N_G-nitro-L-arginine methyl ester (2×10^{-5} M), an inhibitor of the arginine/nitric oxide pathway (Rees et al., 1989) and glibenclamide (2×10^{-6} M), an antagonist of intracellular ATP-sensitive K^+ channels (Cook, 1988; Hassessian et al., 1993). Perfusion pressure and ventricular contraction were continuously recorded.

2.9. Statistics

Results are expressed as mean ± S.E. In these experiments, each heart and each group served as its own control, and responses under control conditions and during specific manipulations were compared in the same heart. For these reasons, statistical significance was determined using a paired *t*-test with a Bonferroni correction factor for multiple comparisons. A statistically significant difference was defined for values of $P \leq 0.05$.

3. Results

3.1. Effects of vasopressin–dextran complexes

Sustained intracoronary infusions of the vasopressin–dextran, (controls 1 and 2; 10^{-7} M) in isolated guinea pig hearts induced no effects in ventricular contraction amplitude nor in perfusion pressure. These results indicate that vasopressin–dextran (control 1) was not active and that there was no non-covalent absorption of vasopressin to dextran–aminocarpoic (control 2).

3.2. Inotropic and vascular effects of vasopressin and vasopressin–dextran

Fig. 1A and B are representative trace recordings of ventricular contraction (upper traces) and perfusion pressure (lower traces) from an isolated perfused guinea pig heart. Infusions of vasopressin or vasopressin–dextran induced a negative inotropic effect and an increase in perfusion pressure (Fig. 1A and B, respectively). Infusion periods are indicated by the arrows. Fig. 1A shows the effects of infusion of 5×10^{-8} M of vasopressin and Fig. 1B shows the effects of 5×10^{-12} M of vasopressin–dextran. Upon termination of infusion the effects of vasopressin or vasopressin–dextran on contraction and perfusion pressure values gradually returned in about 10 min to baseline values; however, the period of washout continued for another 5 min before another dose was given, i.e. there was a 15-min washout period. Using this protocol, the responses to vasopressin and vasopressin–dextran were reproducible. The dose–response curves of the inotropic effects of vasopressin and vasopressin–dextran were done and are shown in Fig. 2A and the corresponding dose–response curves for the vasoconstriction effects are shown in Fig. 2B.

To rule out the possibility that the effects of vasopressin–dextran complex became hydrolyzed to free vasopressin plus dextran–aminocaproic during its passage through the heart, and the observed effects were caused by the released free vasopressin, venous effluent was collected during a 5-min infusion of vasopressin–dextran (10^{-11} M) at two times greater than the concentration the maximal vasopressin–dextran (5×10^{-12} M, Fig. 2). The venous effluent was sieved through a filter with a molecular weight cut-off of 10,000 kD to exclude vasopressin–dextran, and the filtrate which may contain the released vasopressin was infused in the same heart. Infusion of the filtrate had no effect on the ventricular contraction nor

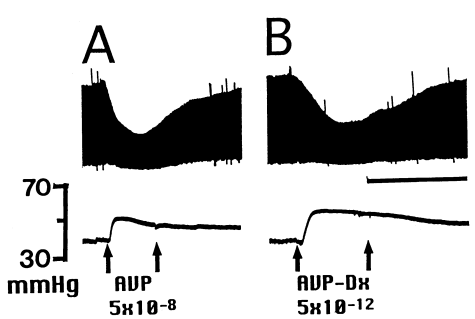

Fig. 1. Effects on ventricular contraction amplitude (upper trace) and perfusion pressure (lower trace) of a sustained coronary infusion of 5×10^{-8} M vasopressin (AVP, part A) and coronary infusion of 5×10^{-12} M vasopressin–dextran (AVP-Dx, part B). Representative traces (A and B) from the same isolated perfused guinea pig heart. Arrows indicate the beginning and the end of the period of infusion. Notice that both vasopressin and vasopressin–dextran cause a negative inotropic and a coronary vasoconstriction effects. In B the time calibration line is equal to 5 min.

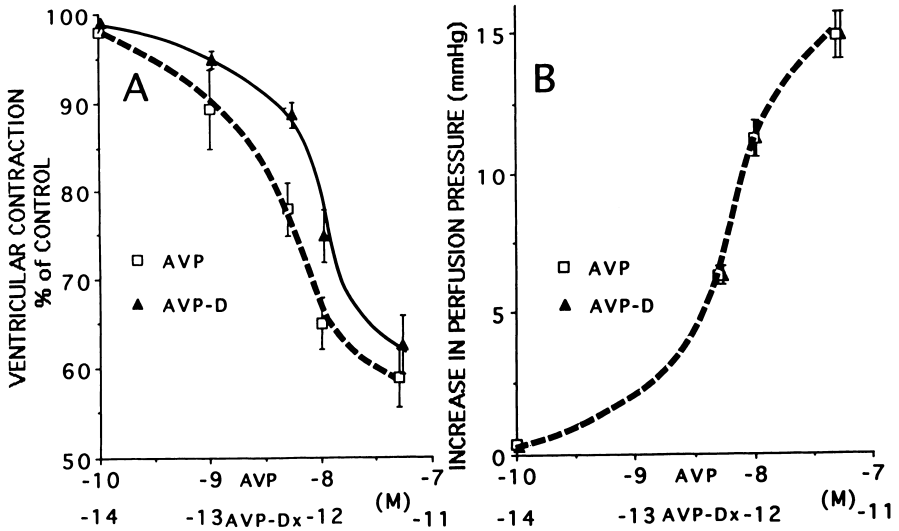

Fig. 2. Dose–response curves of the negative inotropic (ordinates, part A) and coronary vasoconstriction effects (ordinates, part B) caused by coronary infusion of vasopressin (AVP, squares) and vasopressin–dextran (AVP-Dx, triangles). For 10^{-9} M vasopressin and 10^{-13} vasopressin–dextran $n = 5$ and for all the other concentration $n = 21$.

perfusion pressure, showing that there was no free vasopressin present. However, venous effluents containing vasopressin–dextran that were not filtered, upon reinfusion to the heart, caused vasoconstriction and cardiac depression.

The effects of coronary infusion of both vasopressin (5×10^{-8} M) and vasopressin–dextran (5×10^{-12} M), the largest concentrations tested, were blocked by Adam–vasopressin, a vasopressin receptor antagonist, indicating that their effects were vasopressin receptor-mediated (Manning et al., 1987).

3.3. Inhibition or blockade of the effects of vasopressin and vasopressin–dextran

To study possible endothelial mediator mechanism of the inotropic and vascular effects of vasopressin and vasopressin–dextran in the isolated perfused guinea pig hearts, several inhibitors were infused in parallel to vasopressin and vasopressin–dextran. None of the utilized agents with potential blocking effects, when infused alone, had an effect on either ventricular contraction or perfusion pressure. Neither aminophylline, N_G-nitro-L-arginine methyl ester or glibenclamide blocked the ventricular contraction nor the vascular effects of vasopressin and vasopressin–dextran. However, infusion of reactive blue 2, a P_{2y} purinoceptor antagonist (Fieber and Adams, 1991), completely inhibited both the inotropic and vascular effects of vasopressin and vasopressin–dextran (Figs. 3 and 4). Fig. 3(A), (B) and (C) are representative traces of ventricular contraction and perfusion pressure from two different experiments. The blocking effects of reactive blue 2 on the inotropic and vascular effects of vasopressin were exerted with no delay. This is illustrated in Fig. 3(A), where initiation of infusion of reactive blue during the sustained infusion of vasopressin caused an immediate reversal of the inotropic and vascular vasopressin-induced effects. Fig. 3(B) shows the inotropic and vascular effects of a 3-min infusion of vasopressin–dextran (5×10^{-12} M). Initiation of a sustained infusion of reactive blue, 10 s prior to the infusion of vasopressin–dextran (5×10^{-12} M), resulted in a complete blockade of the inotropic and vascular effects of vasopressin–dextran (Fig. 3(C)). Reactive blue reversed the decrease in relative amplitude of the ventricular contraction (Fig. 4(A)) and the increase in perfusion pressure (Fig. 4(B)) induced by vasopressin (5×10^{-8} M)

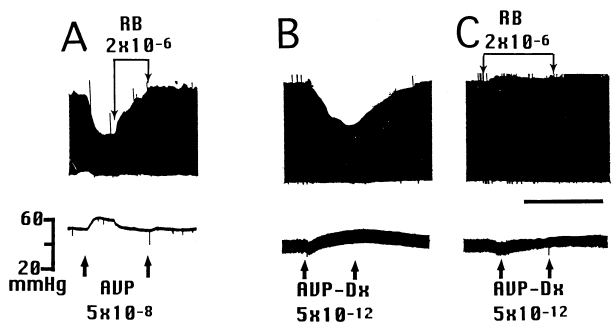

Fig. 3. Blockade by the P_{2y} receptor antagonist reactive blue (RB) of the inotropic and vasoconstriction effects caused by the coronary infusion vasopressin (AVP) and vasopressin–dextran (AVP-Dx). Arrows indicate the beginning and the end of the periods of infusion. (A): rapid reversal of the effects infusion of vasopressin by reactive blue. Infusion of reactive blue was initiated during the sustained infusion of vasopressin. Notice that immediate reversal of the inotropic and vasoconstriction effects of vasopressin. Representative traces from an isolated perfused guinea pig heart. (B) and (C): blockade of the inotropic and vasoconstriction effects of vasopressin–dextran by reactive blue. (B): inotropic and vasoconstriction effects of a maximal concentration of vasopressin–dextran alone. (C): blockade by reactive blue of the effects of vasopressin–dextran when it is infused during a sustained reactive blue infusion. Representative traces from an isolated perfused guinea pig heart. In part C, the time calibration line is equal to 5 min.

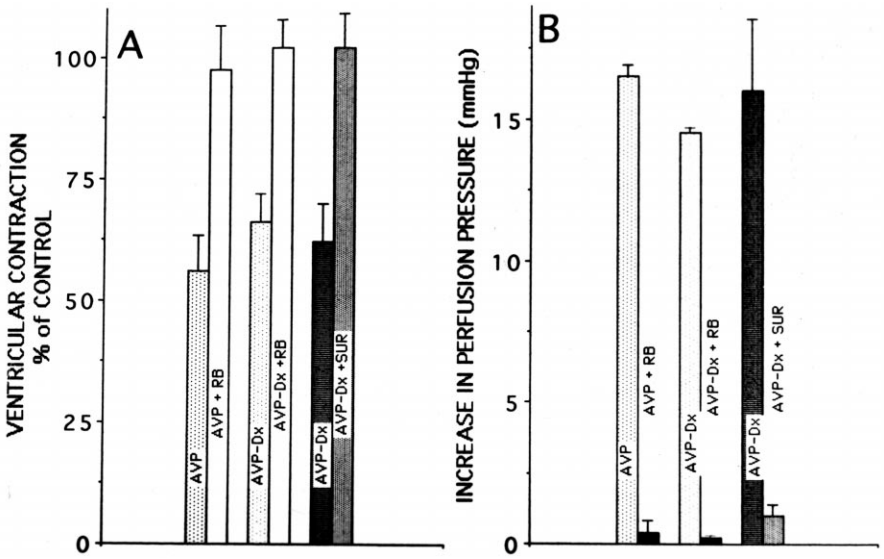

Fig. 4. Reversal by reactive blue (RB, 2×10^{-6} M) and by suramin (SUR, 7.5×10^{-5} M) of the inotropic (A) and vasoconstriction (B) effects caused by the sustained infusion of vasopressin–dextran (AVP-Dx, 5×10^{-12} M) and vasopressin (AVP, 5×10^{-8} M). Only the effects of reactive blue on vasopressin are shown. Columns labelled AVP and AVP-Dx are effects of the agonists alone. Those labelled AVP + RB, AVP-Dx + RB and AVP-Dx + SUR represent infusion of the agonists during the administration of reactive blue or suramin. Notice that administration of reactive blue and suramin resulted in a complete blockade of the inotropic and vasoconstriction effects. Means + SE ($n = 5$).

and vasopressin–dextran (5×10^{-12} M). In the same experimental conditions, pyridoxal-phosphat-6-azophenyl-2′,4′-disulphonic acid (5×10^{-5} M), a P_{2x} receptor antagonist (Zinganshin et al., 1993), did not block the inotropic nor the vascular effects of vasopressin and vasopressin–dextran (not shown). However, suramin (7.5×10^{-5} M), a blocker of both the P_{2y} and P_{2x} receptors (Ohno et al., 1993; Anning et al., 1999), also blocked the inotropic and vascular effects of vasopressin and vasopressin–dextran (Fig. 4(A) and (B)).

4. Discussion

The data presented support the concept that the vascular and ventricular effects, caused by intracoronary infusion of the small-molecular size vasopressin and the large-molecular size vasopressin–dextran, are the result of activation of specific intravascular receptors and suggest that ATP through a P_{2y} receptor activation mediates these effects.

4.1. Activation of intravascular coronary endothelial vasopressin receptors

The controls, dextran–aminocaproic (control 1) and non-covalent adsorption of vasopressin to dextran–aminocaproic (control 2), used in these experiments had no physiologic effect, which demonstrates that the effects of vasopressin–dextran are independent of either reactive chemistry (control 1) or physical adsorption due to retained vasopressin (control 2). Furthermore, we demonstrated that vasopressin–dextran complex is not hydrolyzed to free vasopressin and dextran during its passage through the coronary vasculature (control 3). Coronary venous effluent from hearts perfused with the vasopressin–dextran complex was fractionated by molecular weight exclusion filtering, the low molecular weight fraction when infused in an assay heart, was totally inactive. This demonstrates that there was no free vasopressin in the coronary venous effluent, and that effects of vasopressin–dextran are due to the whole complex. It is concluded that the stability of the vasopressin–dextran complex rests on the formation of the covalent bond between vasopressin and dextran, and that this bond is stable during its passage through the vasculature. Consequently, upon intracoronary infusion of vasopressin–dextran complex there is activation of intravascular coronary endothelial AVP receptors that lead to the observed effects, thus, suggesting that vascular endothelium is a necessary component of vasopressin's vascular and ventricular effects.

Taylor and Granger (1984), using intracoronary perfusion of macromolecular dextran in dog hearts, defined an upper dimensional limit for capillary pore diameter of 0.024–0.032 μm. The dextran used in this study has an estimated molecular diameter of > 0.1 μm (Ceballos and Rubio, 1998). Consequently, the vasopressin–dextran complex could not permeate the capillary wall and, being confined to the intravascular space, could only act on specific endothelial vasopressin receptors.

This study strongly suggests that intracoronary infusion of vasopressin–dextran complex and vasopressin exerts its cardiac actions solely via activation of vasopressin endothelial intravascular receptors. This conclusion is based in the facts that: (a) the effects of both vasopressin and

vasopressin–dextran are blocked by the receptor antagonist Adam–vasopressin (Manning et al., 1987); (b) the vasopressin–dextran complex is chemically stable within the vasculature, and since is too large to cross the endothelium, is confined to the intravascular space; (c) intracoronary infusion of vasopressin and vasopressin–dextran trigger responses which have the same properties, namely, equipotent concentrations of vasopressin and vasopressin–dextran generate the same dose–response curve; and (d) their effects are mediated by the same endothelial chemical messenger (see below). These similarities take place despite that upon intracoronary infusion, vasopressin and vasopressin–dextran could potentially compartmentalize differently. Because of its much smaller molecular size, vasopressin may diffuse freely and occupy intravascular and extravascular compartments and activate receptor at these sites, while vasopressin–dextran activate only intravascular receptors because, being a larger molecule, it is confined to this space.

It is well accepted that hormonal activation of the endothelial lining of blood vessels results in the release of bioactive substances that modulate the tone of adjacent smooth muscle cells (Furchgott and Vanhoutte, 1989). Experimental evidence for this includes multiple studies showing that removal of the blood vessel endothelial lining abolishes or reverses the hormonal response (Furchgott and Vanhoutte, 1989). Working with intact hearts, it is not possible to remove the coronary endothelium without impairing cardiac function. Thus, in order to study endothelium receptor-mediated responses, an appropriate approach is to selectively activate or block endothelial receptors by confining the ligands intravascularly. This protocol has been previously used to study the effects of adenosine by attaching adenosine agonists and antagonist to microspheres and 2000-kD dextran (Balcells et al., 1992, 1993; Rubio et al., 1999), and the effects of acetylcholine by attaching muscarinic receptor antagonist to high-molecular weight dextran (Ceballos and Rubio, 1998).

4.2. The possible endothelial mediator: ATP

It is well known that endothelial cells are capable of rapidly synthesizing ATP (Pearson et al., 1978; Pearson and Gordon, 1985) and is among the list of vasoactive substances released by the endothelium in response to various stimuli (Anning et al., 1999; Hassessian et al., 1993; Ralevic et al., 1992). These findings could help to explain our results with the different P_2 purinoceptor antagonist. We have shown that reactive blue, a P_{2y} purinoceptor antagonist (Fieber and Adams, 1991; Burnstock and Kennedy, 1985), was able to completely and readily revert the inotropic and vascular effects of vasopressin and vasopressin–dextran. Furthermore, simultaneous infusion of 2×10^{-6}-M reactive blue and vasopressin (5×10^{-8} M) or vasopressin–dextran (5×10^{-12} M) completely inhibited their inotropic and vascular effects. Under the same conditions, pyridoxal-phosphat-6-azophenyl-2′,4′-disulphonic acid (5×10^{-5} M), a P_{2x} purinoceptor antagonist (Zinganshin et al., 1993), was without effect. However, suramin, a P_{2x} and P_{2y} purinoceptor antagonist (Ohno et al., 1993), caused the same effects as reactive blue. In addition, in our preparation, the isolated perfused guinea pig ventricular preparation, administration of ATP in a 10^{-7}–10^{-5}-M range caused a concentration-dependent negative inotropic effect (not shown). These results suggest that vasopressin acts on intravascular endothelial receptors and could cause the release of ATP as the sole mediator, which acts via P_{2y} receptors on cardiac and vascular myocytes. However, it is also possible that the vascular and ventricular effects of vasopressin and vasopressin–dextran are the result of a autocrine/paracrine mechanism, in which ATP is just a necessary link between vasopressin endothelial receptor activation and the chosen endpoint (Anning et al., 1999). This is to say that ATP may act in another cell type and not directly on the myocytes. The role of ATP as mediator of hormonal action is not a novel idea. ATP and other nucleotides act as extracellular messengers and exert widespread influence on cell function by acting on a variety of cell surface receptors (Boarder et al., 1995; Harden, 1991).

We are suggesting, and others already have indicated, that extracellular ATP could be a genuine and potent extracellular modulator of cardiac function in ventricular myocardium and in vascular smooth muscle (Anning et al., 1999; Paddle and Burnstock, 1974; Qu et al., 1993). However, it is needed to explain how ATP could depress ventricular contraction and enhance coronary vascular tone. A myriad of diverse pharmacological effects are observed in different tissues in response to adenine nucleotides, and in isolated cardiac cells, the diversity of the responses depends on the source of cardiac tissue type, species and the type of response studied (Fu et al., 1995; Qu et al., 1993; Zhang et al., 1996; Zheng et al., 1993). Despite that in our ventricular preparation, administration of ATP causes a negative inotropic effect which may or may not indicate a direct action on the ventricular myocytes, to accept our proposal it is necessary to demonstrate that ATP causes a negative inotropic effect in freshly isolated guinea pig ventricular myocytes and stimulation of contraction in smooth muscle in coronary resistance vessels. To our knowledge, this information is not available.

In diverse smooth muscle preparations from different species, ATP enhances contraction (Harden, 1991), and in cultured rat aortic smooth muscle cells, activation of P_2 purinoceptors increase intracellular calcium—an effect mediated either by P_{2x} o P_{2y} receptor activation depending on the culture conditions (Pacaud et al., 1995). Thus, it is possible that in our experiments the coronary constriction induced by AVP could be attributed to release of ATP.

In isolated, cultured cardiac myocytes the effects of ATP are diverse. In rat ventricular myocytes, through a P_{2y} purinoceptor activation, ATP causes an increase in Ca^{2+}

influx, intracellular Ca^{2+} and contraction (Zhang et al., 1996; Zheng et al., 1993). In contrast, in isolated cultured ventricular ferret myocytes, ATP causes a negative inotropic effect (Qu et al., 1993), and in the atrial myocytes of most species, ATP activates K + channel activity with an associated depression of contraction (Fu et al., 1995). Unfortunately, studies in isolated, cultured guinea pig ventricular myocytes are not available. Thus, it is possible that ATP in guinea pig ventricular myocytes could cause a negative inotropic effect. The importance of species is stressed by preliminary studies in isolated perfused rat heart, where the effects of vasopressin and vasopressin–dextran are coronary constriction and a positive inotropic effect which are blocked by the P_{2y} purinergic antagonist.

In summary, the available evidence in the literature on the effects of ATP on cardiac ventricular and coronary smooth muscle myocytes does not allow an unequivocal interpretation of our results. However, the strongest evidence still is that the P_{2y}-selective and the P_{2y} and P_{2x} non-selective purinoceptor antagonists both blocked the vascular and inotropic effects of vasopressin or vasopressin–dextran, indicating that ATP is involved in the responses triggered by vasopressin intravascular endothelial receptor activation.

4.3. The lack of effect of other inhibitors tested

All other blockers and inhibitors tested were unable to block the inotropic and vascular effects of vasopressin and vasopressin–dextran. One would have expected that due to the high ectonucleotidase activity in the cardiac cells membranes (Meghji et al., 1992; Pearson et al., 1978), rapid degradation of ATP into adenosine would occur which would produce vasodilation and a negative inotropic effect. However, blockade of the adenosine receptors by aminophylline had no effect on the vascular and inotropic responses to vasopressin or vasopressin–dextran. It has been indicated that ATP-sensitive potassium channels (K^+_{ATP}) which are blocked by glibenclamide are implicated in the release of ATP by flow-stimulated endothelial cells (Hassessian et al., 1993). Furthermore, K^+_{ATP} channels have been implicated in changes in coronary vascular resistance (Melchert et al., 1999). In contrast to these reports, our findings of a lack of blockade by glibenclamide of the vascular and inotropic effects of vasopressin and vasopressin–dextran suggest the mechanism of release of ATP by endothelial cells, in response to vasopressin, does not involve K^+_{ATP} channels. Finally, the lack of action we observed with N_G-nitro-L-arginine methyl ester on the vascular and inotropic effects of vasopressin and vasopressin–dextran indicate that nitric oxide does not participate in this scheme.

4.4. Perspectives

This paper presents evidence that vasopressin, acting at specific intravascular endothelial receptors, induces a decrease in ventricular contraction and an increase in perfusion pressure; both effects require the activation by ATP of P_{2y} purinoceptors. However, the following remain unknown: the defined cell source of ATP, the mechanism of its release, the defined cell target of ATP and the exact mechanism(s) whereby ATP induces these effects (Boarder et al., 1995; Froldi et al., 1999; Qu et al., 1993; Scamps et al., 1992; Seiler et al., 1999; Wilson et al., 1980).

Acknowledgements

This work was supported by grants from the Consejo Nacional de Ciencia y Tecnologia no. 0433P-N, 25963N and 2611PM, and Fundacion Mexicana para la Salud (FUNSALUD).

References

Anning, P.B., Prendergast, B.D., MacCarthy, P.A., Shah, A.M., Buss, D.C., Lewis, M.J., 1999. ATP is involved in myocardial and vascular effects of exogenous bradykinin in ejecting guinea pig heart. Am. J. Physiol. 277, H818–H825.

Balcells, E., Suarez, J., Rubio, R., 1992. Functional role of intravascular coronary endothelial adenosine receptors. Eur. J. Pharmacol. 210, 1–9.

Balcells, E., Suarez, J., Rubio, R., 1993. Implications of the coronary vascular endothelium as mediator of the vasodilatory and dromotropic actions of adenosine. J. Mol. Cell. Cardiol. 25, 693–706.

Boarder, H., Weisman, G.A., Turner, J.T., Wilkinson, G.F., 1995. G protein-coupled P2 purinoreceptors: from molecular biology to functional responses. Trends Pharmacol. 16 (4), 133–139.

Boyle, W.A. III, Segel, L.D., 1986. Direct cardiac effects of vasopressin and their reversal by a vascular antagonist. Am. J. Physiol. 251, H734–H741.

Boyle, W.A. III, Segel, L.D., 1990. Attenuation of vasopressin-mediated coronary constriction and myocardial depression in the hypoxic heart. Circ Res. 66, 710–721.

Burnstock, G., Kennedy, C., 1985. A dual function for adenosine 5′-triphosphate in the regulation of vascular tone; excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. Circ. Res. 58, 319–330.

Ceballos, G., Rubio, R., 1998. Endothelium-mediated negative dromotropic effects of intravascular acetylcholine. Eur. J. Pharmacol. 362, 157–166.

Chiba, S., 1977. Positive chronotropic and negative inotropic actions of vasopressin in isolated blood-perfused canine atrium. Jpn. Heart J. 18, 221–224.

Cook, N.S., 1988. The pharmacology of potassium channels and their therapeutic potential. Trends Pharmacol. Sci. 8, 209–211.

Cowley, A.W., Liard, J.F., 1987. Cardiovascular actions of vasopressin. In: Gash, D.M., DeBoer, G.J. (Eds.), Vasopressin: Principles and Properties. Plenum, New York, NY, pp. 389–433.

DeTar, D.F., Silverstein, R., 1966. Reactions of carbodiimides. II. The reaction of dicyclohexylcarbodiimide with carboxylic acids in the presence of amines and phenols. J. Am. Chem. Soc. 88, 1020–1023.

Fieber, L.A., Adams, D.J., 1991. Adenosine triphosphate-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. J. Physiol. 434, 239–256.

Froldi, G., Gallo, U., Ragazi, E., Caparrotta, L., 1999. 6-benzylaminopurine: a plant derived cytokinin inducing positive inotropism by P_2-purinoreceptors. Planta Med. 65 (3), 245–249.

Fu, C., Pleumsamram, A., Oh, U., Kim, D., 1995. Different properties of the atrial G protein-gated K^+ channels activated by extracellular ATP and adenosine. Am. J. Physiol. 269, H1349–H1358.

Furchgott, R.T., Vanhoutte, P.M., 1989. Endothelium derived relaxing and contracting factors. FASEB J. 3, 2007–2012.

Graf, B.M., Fisher, B., E. Martin, E., Bosnjak, Z.J., Stowe, D.F., 1997. Differential effects of arginine vasopressin on isolated guinea pig heart function during perfusion at constant flow and constant pressure. J. Cardiovasc. Pharmacol. 29, 1–7.

Haga, K., Haga, T., 1983. Affinity chromatography of the muscarinic acetylcholine receptor. J. Biol. Chem. 258 (22), 13575–13579.

Harden, K.T., 1991. P2-purinergic receptor signalling mechanisms. In: Imai, S., Nakazawa, M. (Eds.), Role of Adenosine and Adenine Nucleotides in the Biological System. Elsevier, Amsterdam, pp. 33–41.

Hassessian, H., Bodin, P., Burnstock, G., 1993. Blockade by glibenclamide of the flow-evoked endothelial release of ATP that contributes to vasodilation in the pulmonary vascular bed of the rat. Br. J. Pharmacol. 109, 466–471.

Herlihy, J.T., Bockman, E.L., Berne, R.M., Rubio, R., 1976. Adenosine relaxation of isolated vascular smooth muscle. Am J. Physiol. 230 (5), 1239–1243.

Hupf, H., Grimm, D., Riegger, G.A.J., Schunkert, H., 1999. Evidence for a vasopressin system in the rat heart. Circ. Res. 84, 365–370.

Katusic, Z.S., Shepherd, J.T., Vanhoutte, P.M., 1984. Vasopressin causes endothelium-dependent relaxations of the canine basilar artery. Circ. Res. 55, 575–579.

Manning, M., Przybylski, J.P., Olma, A., Klis, W.A., Kruszynski, N.C., Wo, N.C., Pelton, G.H., Sawyer, W.H., 1987. No requirements of cyclic conformation of antagonist in binding to vasopressin receptors. Nature 329, 839–840.

Marttila, M., Voulteenaho, O., Ganten, D., Nakao, K., Ruskoaho, H., 1996. Synthesis and secretion of natriuretic peptides in the hypertensive TGR(mREN-2)27 transgenic rat. Hypertension 28, 995–1004.

Meghji, P., Pearson, J.D., Slakey, L.L., 1992. Regulation of extracellular adenosine production by ectonucleotidases of adult rat ventricular myocytes. Am J. Physiol. 263, H40–H47.

Melchert, P.J., Dunker, D.J., Traverse, J.H., Bache, R.J., 1999. Role of K^+_{ATP} channels and adenosine in regulation of coronary blood flow in the hypertrophied left ventricle. Am. J. Physiol. 277, H617–H625.

Michell, R.H., Kirk, C.J., Billah, M.M., 1979. Hormonal stimulation of phosphatidylinositol breakdown with particular reference to the hepatic effect of vasopressin. Biochem. Soc. Trans. 7, 861–865.

Moalic, J.M., Bauters, C., Himbert, D., Bercovici, J., Mouas, C., Guicheney, P., Baudoin-Legros, M., Rappaport, L., Emanoil-Ravier, R., Mezger, V., 1989. Phenylephrine, vasopressin and angiotensin as determinants of protooncogene and heat-shock protein gene expression in adult rat heart and aorta. J. Hypertens. 7, 195–201.

Murase, T., Kozawa, O., Miwa, M., Tokuda, H., Kotoyori, J., Kondo, K., Oiso, Y., 1992. Regulation of proliferation by vasopressin in aortic smooth muscle cells: function of protein kinase C. J. Hypertens. 10, 1505–1511.

Ohno, N., Ito, K.M., Yamamoto, Y., Suzuki, H., 1993. Suramin selectively inhibits the no-adrenergic, non-cholinergic inhibitory junction potential in guinea pig stomach. Eur. J. Pharmacol. 2499, 121–128.

Pacaud, P., Malam-Souley, R., Loirand, G., Desgranges, C., 1995. ATP raises $[Ca^{2+}]_i$ via different P2-receptor subtypes in freshly isolated and cultured aortic myocytes. Am. J. Physiol. 269, H30–H36.

Paddle, B.M., Burnstock, G., 1974. Release of ATP from perfused heart during coronary vasodilation. Blood Vessels 11, 110–119.

Pearson, J.D., Carleton, J.S., Hutchings, A., Gordon, J.L., 1978. Uptake and metabolism of adenosine by pig aortic endothelial and smooth muscle cells in culture. Biochem. J. 178, 265–271.

Pearson, J.D., Gordon, J.L., 1985. Nucleotide metabolism by endothelium. Annu. Rev. Physiol. 47, 617–627.

Qu, Y., Himmel, H.M., Campbell, D.L., Strauss, H.C., 1993. Effects of extracellular ATP on I_{Ca}, $[Ca^{2+}]_i$, and contraction in isolated ferret ventricular myocytes. Am. J. Physiol. 264, C702–C708 (Cell Physiol. 33).

Ralevic, V., Lincoln, J., Burnstock, G., 1992. Release of vasoactive substances from endothelial cells. In: Ryan, U.S., Rubany, G.M. (Eds.), Endothelial Regulation of Vascular Tone. Marcell Dekker, New York, pp. 297–328.

Rees, D.D., Palmer, R.M., Hodson, H.F., Moncada, S., 1989. Specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. Br. J. Pharmacol. 96, 418–424.

Rubio, R., Ceballos, G., Balcells, E., 1999. Intravascular adenosine: the endothelial mediators of its negative dromotropic effects. Eur. J. Pharmacol. 370, 27–37.

Scamps, F., Rybin, V., Pugeat, M., Tkachuk, V., Vassort, G., 1992. A G2l protein couples P_2 purinergic stimulation to cardiac Ca^{++} channels without cyclic AMP production. J. Gen. Physiol. 100, 675–698.

Seiler, L., Matyas, S., Fleckenstein-Grun, G., 1999. Extracellular ATP increases $[Ca^{2+}]_i$, in primary cultured pig coronary smooth muscle cells via P_{2y} purinoreceptor subtype. J. Cardiovasc. Pharmacol. 33 (5), 807–813.

Swords, B.H., Wyss, J.M., Berecek, K.H., 1991. Vasopressin and vasopressin receptors are enhanced in the central nervous system in deoxycorticosterone-NaCl hypertension. In: Jard, S., Jamison, R. (Eds.), Vasopressin. John Libbey Eurotext, Paris, France, pp. 409–417.

Taylor, A.E., Granger, D.N., 1984. Exchange of macromolecules across the microcirculation. In: Renkin, E.M., Michel, C.C., Geiger, S.R. (Eds.), Handbook of Physiology. Section 2. The Cardiovascular System. Vol. 4 Microcirculation Part 1. Am. Phys. Soc. vol. 4, pp. 488–510, Bethesda.

Wilson, M.F., Brackett, D.J., Archer, L.T., Hinshaw, L.B., 1980. Mechanisms of impaired cardiac function by vasopressin. Ann. Surg. 191, 494–500.

Zhang, B.-X., Ma, X., McConnell, B.K., Damron, D.S., Bond, M., 1996. Activation of purinergic receptors triggers oscillatory contractions in adult rat ventricular myocytes. Circ. Res. 79, 94–102.

Zheng, J.-S., Christie, A., Levy, M.N., Scarpa, A., 1993. Modulation by extracellular ATP of two distinct currents in rat myocytes. Am. J. Physiol. 264, C1411–C1417.

Zinganshin, A.U., Hoyle, C.H.V., Xuenong, B., Lambrecht, G., Mutschler, E., Baumert, H.G., Burnstock, G., 1993. PPADS selectively antagonizes P_{2x} purinoreceptor-mediated responses in the rabbit urinary bladder. Br. J. Pharmacol. 110, 1491–1498.